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Low Fouling Thin Hydrogel Coatings Made of Photo-crosslinked Polyzwitterions

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Abstract

Although zwitterionic chemistries are among the most promising materials for producing non-fouling surfaces, their structural diversity has been small up to now. Here, we compare the *in vitro* fouling behavior of a set of four systematically varied sulfa-/sulfobetaine-containing zwitterionic hydrogel coatings against a series of proteins and non-motile as well as motile marine organisms as model foulers. The coatings are prepared by simultaneous photo-induced crosslinking and surface anchoring, to elucidate the effect of the molecular structure of the zwitterionic moieties on their antifouling activity. Analogously prepared coatings of poly(butylmethacrylate) and poly(oligoethylene glycol methacrylate) serve as references. Photo-reactive polymers are synthesized by statistical copolymerization of sulfobetaine or sulfabetaine methacrylates and methacrylamides with a benzophenone derivative of 2-hydroxyethylmethacrylate and applied as thin film coating. While keeping the density of the zwitterionic and of the crosslinker groups constant, the molecular structure of the zwitterionic side chains is varied systematically, as is the arrangement of the ion pairs in the side chain by changing the classical linear to a novel Y-shaped geometry. All the polyzwitterions strongly reduce fouling compared to poly(butylmethacrylate). Overall, the sulfabetaine polyzwitterion coatings studied even match the high anti-fouling effectivity of oligo(ethylene glycol)-based ones used as control. Nevertheless, performances varied individually for a given pair of polymer and fouler. The case of the polysulfobetaines exemplifies that minor chemical changes in the polymer structure affect the antifouling performance markedly. Accordingly, the antifouling performance of such polymers cannot be correlated simply to the type of zwitterion used (which could be generally ranked as "better performing" or "less performing"), but is a result of the polymer's precise chemical structure. Our findings underline the need to enlarge existing

structural diversity of polyzwitterions for anti-fouling purposes, to optimize the potential of their chemical structure.

Introduction

Biofouling is responsible for severe economic, health and ecological damage, e.g. in biomedical, membrane and marine applications.[1,2] It is a major challenge to fight this problem with environmentally benign coatings which are free of biocides.[3–5] Since biocidal compounds have been increasingly banned for marine applications, new polymers for antifouling-coatings need to be developed.[6] Several studies have shown that charge neutrality at the interface, steric repulsion, and hydration of such polymers are guiding design rules for inert surfaces.[7–10] Among potential candidates for future antifouling coatings, zwitterionic systems appear particularly attractive as they are highly resistant against protein adsorption[11–14] and against many freshwater and marine fouling organisms.[15–18] Systematic studies of self-assembled monolayers as model surfaces revealed the relevance of charge neutrality and the selection of anionic and cationic groups.[8,17,19] In particular the sulfonate moiety was identified as promising anionic group in zwitterions for inert coatings.[8,17,19]

While model surfaces provide well-controlled surface chemistries, the zwitterionic functionality needs to be incorporated into polymer coatings for real applications.[20] An established route for synthesizing low-fouling polymer coatings is through surface grafting reactions. In particular, zwitterion-functionalized polymer brushes, e.g. made of polyacrylates and polymethacrylates, can be anchored to surfaces by grafting-from, grafting-to, and grafting-through using covalent linkage or living polymerization reactions.[21] Alternatively, thin film polymer coatings can be prepared by spreading of the polymers onto surfaces with subsequent crosslinking.[22–24] Crosslinkers can additionally serve to integrate monomers into polymers,[25] and if tethered to surfaces, as anchor groups for polymer immobilization.[26,27] An alternative way is the use of copolymers that contain inherent crosslinker moieties such as (4-

benzoylphenoxyl)-functionalized methacrylates.[28–32] After coating the polymers onto surfaces (e.g. via spin coating), the benzophenone can be conveniently excited by near-UV light to initiate the crosslinking reaction via abstraction of aliphatic hydrogens and subsequent recombination of the radicals formed.[30,33] Because of the low specificity of this reaction it can be applied to most organic polymers and it enables simultaneous crosslinking of the polyzwitterionic chains as well as the covalent anchoring of the polymer network produced to the substrate.[34]

In previous reports, the remarkable antifouling properties of polyzwitterions have been recognized. For instance, Jiang et al reported the inertness with respect to protein adsorption and barnacle cyprid settlement of carboxy- and sulfobetaine methacrylate (CBMA and SBMA, respectively) polymer brushes.[4,18] Colak et al compared different polybetaine copolymers and discovered a protein resistant class of fluorine-containing amphiphilic polybetaines.[35] Lange et al used a sulfobetaine systems equipped with a clickable azide moiety[36] which effectively resisted nonspecific protein adsorption, while Rodriguez-Emmenegger et al used grafted zwitterionic brushes synthesized via single electron transfer-living radical polymerization to suppress platelet adhesion.[37] In the field of biofilm formation, Cheng et al showed that the formation of freshwater biofilms of *Pseudomonas putida* and *P. aeruginosa* is reduced by a poly(carboxybetaine methacrylate).[15] Yang et al demonstrated the effectiveness of zwitterions against green algae and marine diatoms by using polyvinyl alcohols functionalized with sulfobetaines.[25,38] Rather hydrophobic coatings of lauryl methacrylate SPE and CBMA with low zwitterion content showed only a minor improvement in fouling-release performance against *Navicula incerta*.[39] In contrast, SPE and CBMA polymers with high zwitterion content showed very good fouling release performance against *Navicula incerta*.[40] The incorporation of zwitterionic SBMA groups into siloxane-polyurethane coatings improved their fouling-release properties.[41] A comparison of zwitterionic polymers with a poly(arylene ether sulfone),

a polyacrylate and a surface grafted polymethacrylate backbone revealed the relevance of the polymer matrix for the fouling-release effect of betaine moieties.[42]

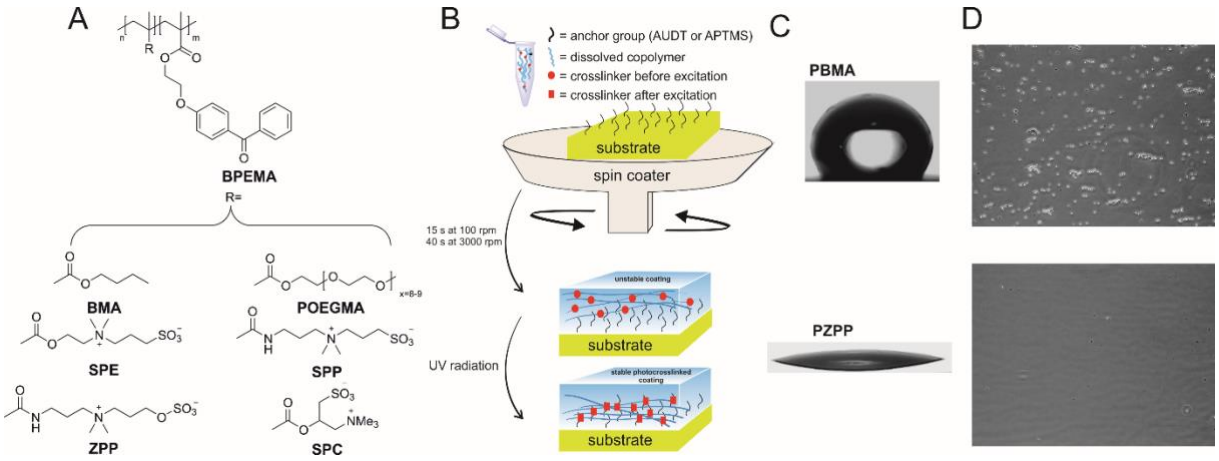

Figure 1. (A) Structures and abbreviations of the copolymers investigated, using as main building blocks butyl methacrylate (BMA), oligo(ethylene glycol) methacrylate (OEGMA), N-(2-methacryloyloxy)ethyl-N,N-dimethylammonio propanesulfonate (SPE), N-(3-methacryloylimino)propyl-N,N-dimethyl-ammonio propanesulfonate (SPP), 3-((3-methacrylamidopropyl)dimethylammonio)propyl sulfate (ZPP) and 2-(methacryloyloxy)-3-(trimethylammonio)propane-1-sulfonate (SPC). Small amounts (1.0 mol%) of methacrylate BPEMA were incorporated as photoreactive group to enable crosslinking of the spin-coated films. (B) Schematic representation of the preparation of the coatings: the copolymers were spin-coated on amino terminated substrates. UV radiation yields stable photocrosslinked coatings. (C) Static water contact angle of coatings of the hydrophobic non-ionic PBMA (top) and the hydrophilic zwitterionic PZPP (bottom). (D) *Navicula perminuta* (white dots) on the polymer coatings made of BMA (top) and ZPP (bottom) after a 90 min fluidic assay.

While the excellent non-fouling properties of zwitterionic polymers are undisputed, studies are nearly exclusively limited to polymers containing the few commercially available propanesulfonate zwitterions.[20] A systematic understanding of the optimized design of the polymer backbone and the spatial arrangement of the oppositely charged moieties for protein

resistance and marine fouling-release applications is still lacking. In this work, we address this gap and explore a series of photo-cross-linkable copolymers of zwitterionic sulfo- and sulfabetain methacrylates with 2-(4-benzoylphenoxy)ethyl methacrylate (BPEM) to prepare low-fouling coatings. A set of systematically varied monomers was used for the copolymers in order to screen how the nature of their building blocks affects the antifouling properties of zwitterionic coatings (Figure 1A). Namely, we compare polymers based on the frequently employed sulfobetaines *N*-(2-methacryloyloxy)ethyl-*N*,*N*-dimethylammonio propanesulfonate (SPE) and *N*-(3-methacryloylimino)propyl)-*N*,*N*-dimethyl-ammonio propanesulfonate (SPP), that differ in the connection of the zwitterionic motif to the polymer backbone via an ester group (methacrylate) or an amide group (methacrylamide), respectively, and in the length of their aliphatic spacer groups separating the zwitterions from the backbone. In addition, we compare the polymer of the new sulfobetaine ester 2-(methacryloyloxy)-3-(trimethylammonio)propane-1-sulfonate (SPC). In contrast to its analogue SPE, the methacrylate group is attached in SPC directly to the betaine motif without any spacer, right in the center of the trimethylene group that separates the anionic and the cationic groups of the ammoniopropanesulfonate moiety. While the zwitterionic moiety is attached perpendicularly to the polymer backbone in the case of PSPE, the zwitterionic moiety is attached in parallel to the polymer backbone for PSPC and may assume a Y-shape, so that the cationic and anionic group are placed at the same distance from the polymer backbone. Moreover, we employ in our study as alternative zwitterionic component the methacrylamide *N*-(3-methacrylamidopropyl)-*N*,N-dimethylammonio propyl sulfate (ZPP). ZPP is an analogue of SPP, in which the anionic sulfonate group of the zwitterion in the latter is replaced by a sulfate group.[21] This polyzwitterion class that was named "polysulfabetaine" has received little attention up to now.[43–46] Although one modeling study suggested that polysulfabetaines should not perform well in antifouling systems[47], the prediction has been only sparsely verified experimentally to our knowledge.[20] Also, we included the nonionic copolymers of butylmethacrylate (BMA) or oligo(ethylene glycol)

methacrylate (OEGMA) that served as negative and positive controls, respectively. All copolymers were molecularly characterized by elemental analysis, ^{1}H-NMR spectroscopy, and size exclusion chromatography (SEC). The copolymers were applied as thin film coatings (≈ 140 nm thickness) by spin coating on aminosilane or aminothiol functionalized substrates (Figure 1B). Subsequently the films were crosslinked by UV radiation, and the antifouling properties of the obtained thin hydrogel films were tested against protein adsorption, namely of bovine serum albumin (BSA), fibrinogen, and lysozyme. Furthermore, the best performing polymers were tested as protective coatings against diatom *Navicula perminuta*, attachment (Figure 1D) and settlement of zoospores of the green alga *Ulva linza*.

Materials and Methods

Chemicals. Trifluoroethanol (>99.8 %, Carl Roth, Karlsruhe/Germany), ethanol (Chemsolute / Th. Geyer, Renningen/Germany, ≥99.5 %), acetone (HPLC Grade 99.5+ %, Alfa Aesar), chloroform, cyclohexane, ethanol, ethylacetate, toluene (all analytical reagent grade, Fisher Scientific), glutaraldehyde solution 25% (Fisher Scientific, certified grade) and nitrogen (99%, Air Liquide), octadecyltrichlorosilane (> 90 %, Sigma Aldrich), 3-aminopropyltrimethoxy silane (APTMS; 97 %, Sigma Aldrich), and 11-amino-1-undecanethiol (AUDT, ProChimia Surfaces) were used as received. Initiator 2,2'-azobisisobutyronitrile (AIBN, Sigma Aldrich, 98 %) was crystallized from methanol prior to use. Butyl methacrylate (Sigma Aldrich, containing 10 ppm of monomethylhydroquinone "MEHQ" as inhibitor) was purified prior to use by distillation. Poly(ethylene glycol) methyl ether methacrylate (OEGMA, M_r=500, Sigma Aldrich, containing 100 ppm of monomethylhydroquinone "MEHQ" and 200 ppm of 2,6-di-*tert*-butyl-4-methylphenol "BHT" as inhibitors) was purified prior to use by filtration through a bed of aluminum oxide (ALOX 90 active neutral, 70-230 mesh ASTM 0.063-0.2mm, Merck). Monomers 3-[N-2-(methacryloyloxy)ethyl-N,N-dimethyl]ammonio propane-1-sulfonate (SPE, Sigma Aldrich, ≥ 97%) and 3-[N-3-(methacrylamido)propyl-N,N-dimethyl]ammonio propane-

1-sulfonate (SPP, gift from Raschig GmbH) were used as received. Zwitterionic monomers were synthesized and characterized as described elsewhere.[30,48] 2-(4-benzoylphenoxy)ethyl methacrylate (BPEMA, gift from Michael Päch, IAP Fraunhofer Potsdam/Germany). Albumin from bovine serum (BSA, ≥98 %, Sigma Aldrich), fibrinogen from bovine plasma (Sigma Aldrich) and lysozyme from chicken egg white (Sigma Aldrich) were used as received. Ultrapure water was purified with a milliQ system (Siemens Water Technology, Germany). Model salt water (SW) for the stability tests was freshly prepared using the ingredient protocol of Kester.[49] Filtered sea water (FSW, CaribSea, Florida, USA) and artificial sea water (ASW, Tropic Marin® salts) were used as received.

Polymer synthesis. *n*-Butyl methacrylate (BMA) was copolymerized with 2-(4-benzoylphenoxy)ethyl methacrylate (BPEMA) and azobisisobutyronitrile (AIBN) in bulk. AIBN (33.4 mg, 0.2 mmol, 0.01 equivalent) and BPEMA (62.5 mg, 0.2 mmol, 0.01 equivalent) were dissolved in freshly distilled BMA (2.85 g, 20 mmol) at ambient temperature. The mixture was purged with nitrogen for 45 min to remove oxygen, before it was heated to 70 °C under inert atmosphere for 20 h while continuously stirring. By cooling and exposing the mixture to the atmosphere, the polymerization was stopped. The highly viscous mixture was diluted by 30 ml of acetone, and precipitated into 100 ml of methanol. The supernatant liquid was decanted, and the residue washed by methanol. Purification by precipitation was repeated, before the residue is dried at 35°C in vacuo. The polymer was obtained as colorless powder (2.26 g, yield 77 %).

Zwitterionic polymers were synthesized via free radical copolymerization in solution following a standard procedure.[48] 20 wt% of zwitterionic monomer, 2-(4-benzoylphenoxy)ethyl methacrylate (BPEM, 1.0 mol% relative to monomer) and azobisisobutyronitrile (AIBN, 0.5 mol% relative to monomer) were dissolved in trifluoroethanol (TFE, 78 wt%) and purged with argon for 30 min at ambient temperature to remove oxygen. The reaction mixture was

polymerized under the inert gas at 60 °C for 20 h. Then, by cooling the mixture and exposure to air, polymerization was terminated. After dilution by ultrapure water, the mixture is dialyzed against ultrapure water, using a membrane type ZelluTrans (Roth, Germany) with a nominal molecular weight cut off MWCO of 3,500 g mol^{-1}, and lyophilized. The polyzwitterions were obtained as hygroscopic colorless solids. Inspired by a reported procedure,[30] solution copolymerization of OEGMA and BPEMA was conducted as described above, but replacing the reaction solvent TFE by ethanol. Details of polymer preparation and characterization are listed in Table 1.

Table 1. Polymer preparation and characterization.

Monomer	BPEM [mol%]	Solvent	Yield [%]	Content of BPEM [mol%][(a)]	M_n^{app} [kg mol^{-1}][(b)]	Dispersity Đ[(b)]
PBMA	1.0	-[(c)]	56	1.0 ± 0.3	50[(d)]	3.2[(d)]
POEGMA	1.0	EtOH	82	1.0 ± 0.3	260[(e)]	2.9[(e)]
PSPE	1.0	TFE	72	1.0 ± 0.3	90[(f)]	5.4[(f)]
PSPP	1.0	TFE	75	1.0 ± 0.3	180[(f)]	2.3[(f)]
PZPP	1.0	TFE	79	1.1 ± 0.3	190[(f)]	3.6[(f)]
PSPC	1.0	TFE	23	1.1 ± 0.3	60[(f)]	3.3[(f)]

[(a)] determined by NMR; [(b)] apparent number average molar mass M_n^{app} and dispersity Đ (M_w^{app}/M_n^{app}) by SEC; [(c)] bulk polymerization; [(d)] eluent THF, calibration with linear polystyrene standards; [(e)] eluent NMP, calibration with linear polystyrene standards; [(f)] eluent HFIP, calibration with linear poly(methylmethacrylate) standards.

Glass-octadecyltrichlorosilane (OTS) preparation. Glass slides (Nexterion Slide Glass B, Schott), were activated in an oxygen plasma (0.4 mbar, 80 W, 22 kHz, miniFlecto-PC-MFC, Gala Instruments GmbH) for three minutes. The cleaned and activated slides, were inserted

under nitrogen into a custom build silanization reactor, which was filled with 0.5 mM solution of octadecyltrichlorosilane in a cyclohexane chloroform mixture (v/v, 75:25). After 30 min in an ice cooled ultrasonic bath, the slides were sonicated for 30 s in cyclohexane and 30 s in toluene.

Sample preparation. To enable a stable attachment of the polymers to the surface, the glass and silicon substrates were modified with an aminosilane adhesion promoting monolayer and then coated with the polymer. For the bioassays, glass slides (Nexterion Slide Glass B, Schott) were modified with 3-aminopropyltrimethoxy silane (APTMS) following established protocols[50,51]. The slides were activated in an oxygen plasma (0.4 mbar, 80 W, 22 kHz, miniFlecto-PC-MFC, Gala Instruments GmbH) for 3 min before immersing them into a 5% (v/v) APTMS solution in acetone under inert gas (nitrogen) atmosphere for 30 mins. Subsequently, the samples were sonicated for 20 s in ethylacetate, 20 s in ethanol, and 20 s in milliQ water.

For protein resistance assays using SPR and for surface analysis, gold-coated glass slides (D263, Schott, coated with a 5 nm titanium adhesion promoter and a 100 nm thick gold film) were used.[52,53] 11-amino-1-undecanethiol (AUDT) monolayers were assembled as previously described from a 1 mM solution in ethanol.[54] First, the gold chips were cleaned under UV light for 1 h, followed by rinsing and sonicating three times in ethanol for 3 min. The samples were then immersed for 24 h in the solution of AUDT in ethanol. Afterwards, the samples were rinsed and sonicated for 3 min in ethanol and dried in a stream of nitrogen.

The substrates were coated with the polymer films by spin coating (WS-650MZ-23NPP/Lite, Laurell Technologies Corporation). To spread the polymer solution on the surface, 35 µL of the polymer dissolved in TFE (1 wt%) were deposited on the SPR chips and 500 µL were deposited on the objective slides for the bioassays during spinning of the substrates for 15 s at 100 rpm. Subsequently, the spinning speed was increased to 3000 rpm for 40 s to remove excess material

and to reach the desired film thickness of 140 nm ±10 nm. The surfaces were then exposed to UV radiation for 30 min. (Uvacube 100, Dr. Hönle AG, 100 W iron doped mercury vapour lamp, H1 filter with cut off >310 nm, distance to sample 18 cm, intensity at sample 15 mW/cm²) After the coating process, the samples were immersed for 10 s three times in distilled water to remove unattached polymer chains or monomers and to allow the film to restructure under water.

Spectroscopic ellipsometry. Dry film thicknesses were determined by using spectroscopic ellipsometry (M-2000, Woollam, USA; CompleteEase software package) at three angles (65°, 70°, 75°) relative to the surface. The thickness of the organic layers (SAMs, APTMS) was determined by using a transparent single layer model with a wavelength depending refractive index described by the Cauchy model (A = 1.45, B = 0.01). The thickness of the spin coated polymer coatings was modelled by a non-transparent layer (absorbing film) model using a ß-spline to describe the wavelength dependent refractive index and absorption coefficient. For each sample, three different points were measured and averaged.

Static water contact angle goniometry. Static water contact angles were measured using a custom-built goniometer. Droplets of tridistilled water were dispensed on the surface and recorded by a CCD camera. The droplet shape was fitted by using Young's equation to determine the contact angle. For each sample, three different points were measured and averaged.

Stability test. To test the stability of the polymer in aqueous solution, polymers on gold coated slides were immersed for 1 h, 2 h, 1 d, 2 d, 3 d and 7 d in saltwater. This salt solution was freshly prepared, had a pH value of 8 and was used rather than artificial sea water (ASW) to avoid the formation of conditioning layers on the polymer coatings[54]. The samples were shaken for 1 min in milliQ water to remove excess salt from the surface, before measuring the film thickness *via* spectroscopic ellipsometry.

ATR-FTIR spectroscopy. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were recorded using a Bruker Tensor 27 spectrometer equipped with a liquid nitrogen-cooled MCT detector and a Ge-ATR-crystal. Prior to the measurements, the sample compartment and the MCT chamber were purged with nitrogen for 30 minutes.

Atomic force microscopy (AFM). AFM images were measured with a apparatus NanoWizard 3 (JPK). The scan of the images was defined by 10 µm x10 µm with a 4 V target amplitude, 2 V setpoint, 750 Hz i-gain and 89 µm/s tip velocity. The root mean square value was determined by using the tools of the freeware Gwyddion.[55]

X-ray Photoelectron Spectroscopy (XPS). XPS spectra were recorded by an electron spectrometer equipped with a hemispheric analyzer (Type CLAM2, VG, Scientific, Thermo Fischer Scientific) and a polychromatic aluminum anode ($E(Al\ K_\alpha) = 1486$ eV).

Protein adsorption by surface plasmon resonance spectroscopy. Protein adsorption was measured by surface plasmon resonance spectroscopy (SPR) (SR7000DC, pump SR7500, autosampler SR7100, Reichert) by using gold-coated thin glass slides (D263, Schott coated with a 5 nm titanium adhesion promoter and a 100 nm thick gold film)[52,53]. The SPR chip was attached to the prism using refractive index matching fluid (Cargille, Immersion Oil, Type A) for good optical transmission. The channel on top of the SPR chip was rinsed with PBS at a flow rate of 200 µL/min until a stable baseline was obtained. 1000 µl of protein solution (1 mg/mL in PBS) was pumped across the surface at a flow rate of 10 µL/min for 10 min before switching to PBS buffer at the same flow rate for 15 minutes. The resulting shift of the SPR signal before protein injection and after the 15 min PBS rinsing step is reported as irreversible protein attachment.

Dynamic Navicula perminuta attachment assay. The parallel dynamic microfluidic accumulation assay of diatoms was performed following previously published protocols.[56] From a continuous culture of the marine diatom *Navicula perminuta*, a suspension with a cell

concentration of 3 million/mL in sea water SW, was prepared. After an overnight preincubation of the coated glass slides in salt water, IBIDI sticky slides 0.1 (IBIDI) were fixed onto the samples to create a microfluidic environment. The assay included a dynamic 90 min accumulation experiment in which the diatom suspension was pumped across the surface at a constant wall shear stress of 0.14 Pa followed by a 10 min rinsing step at the same wall shear stress with pure salt water to remove excess diatoms from the channel and tubings. To quantify diatom attachment, thirty fields of view were recorded in the central part of the channel by phase contrast microscopy (Nikon Eclipse TE2000-U, 10x phase contrast objective Nikon CFI Plan Fluor DLL NA 0.3, Nikon). Diatoms were counted automatically by using NIS Elements software (Nikon). Observed differences were tested by an ANOVA analysis with posthoc Tukey test (significance level $p = 0.05$) with regard to statistical significance.

Settlement of zoospores of the green alga Ulva linza. Fronds of *U. linza* were collected from Craster, UK (55°26′ N; 1°35′ W) and a spore suspension of 1.0×10^6 spores ml^{-1} was prepared by the method of Callow et al.[57] Three replicates of each sample, prepared as coatings on glass slides (preincubated in 0.22 μm-filtered ASW for 24 hours), were placed in individual compartments of quadriPERM® dishes. Ten ml of spore suspension were added to each well and the dishes were incubated in darkness at approximately 20°C. After 45 min, the slides were washed by passing backward and forwards ten times through a beaker of seawater to remove unsettled (i.e. swimming) spores. Slides were fixed in 2.5% glutaraldehyde in ASW. The density of settled zoospores was counted on each slide using a Zeiss Axioscope fluorescence microscope fitted with Leica LAS X image analysis software. Cells were located by the autofluorescence of chlorophyll contained within them. Counts were made for 30 fields of view (each 0.15 mm^2) on each slide. Observed differences between surfaces were tested by an

ANOVA analysis with posthoc Tukey test (significance level p = 0.05) with regard to statistical significance.

Results and Discussion

Surface Characterization.

Table 2. Static water contact angle and RMS roughness of the different polymer coatings as determined by AFM. Errors represent the standard deviation (n = 10).

	Water contact angle	RMS roughness
PBMA	80° ±3°	71.5 Å
POEGMA	37° ±1°	3.5 Å
PSPE	26° ±2°	2.5 Å
PSPP	21° ±2°	2.3 Å
PZPP	24° ±2°	3.7 Å
PSPC	20° ±2	2.5 Å

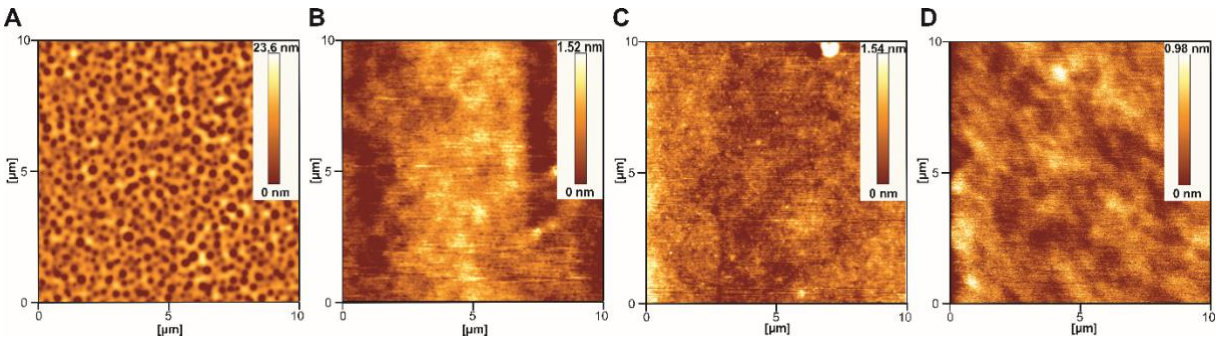

Figure 2. Representative AFM images (10 µm x 10 µm) for determining the RMS values and surface morphologies. Images show coatings of PBMA (A), POEGMA (B), PSPE (C), and PSPP (D). Note the different z-ranges which go up to 23.6 nm (A), 1.52 nm (B), 1.54 nm (C) and 0.98 nm (D).

Copolymers from the monomers in figure 1A and the photo-crosslinkable benzophenone methacrylate monomer were obtained by free radical statistical copolymerization and applied as thin film coatings by spin coatings. The general use of methacrylic polymerizable groups for all components is expected to provide a largely homogeneous, random distribution of the crosslinking sites within the polymers. After photo−crosslinking with UV radiation and a short leaching step, the surfaces were characterized by surface analysis. Water contact angle goniometry was used to characterize the wettability, which is generally assumed to have a significant influence on the antifouling behavior of coatings, of the prepared polymer (figure 1C, table 2). Films of the uncharged hydrophobic PBMA reference had a static water contact angle (WCA) of 82°, while the WCA of the oligo(ethylene glycol)-containing coatings reached 37°. All zwitterionic coatings were more hydrophilic compared to PBMA and POEGMA (WCA < 30°), whereas only minor differences were found between the various polyzwitterions. PSPE (26°) and PZPP (24°) seemed slightly less hydrophilic than PSPP (21°) and PSPC (20°), but all differences were close to the size of the error bars. Interestingly, the Y−shaped zwitterionic arrangement yielded the most hydrophilic surfaces. To characterize the roughness of the samples, 10 x 10 μm^2 large AFM images (Figure 2) were recorded to see if spin coating is a suitable method to apply the copolymers to the surface. The RMS values were calculated and can be seen in table 1. The zwitterionic and the POEGMA-coatings were very smooth, their RMS values varying between 2.3 Å and 3.7 Å, confirming that spin coating is a suitable method for applying the copolymer to the surface. In contrast, the RMS value of 71.5 Å of the PBMA coating was much higher as compared to the polyzwitterions. The high roughness might be due to the consistent use of trifluoroethanol as solvent. This could induce roughness during the drying phase because of the lower solubility of PBMA in trifluoroethanol. However, the higher contact angle of PBMA should have its origin in the nature of chemistry and not in the higher RMS value.

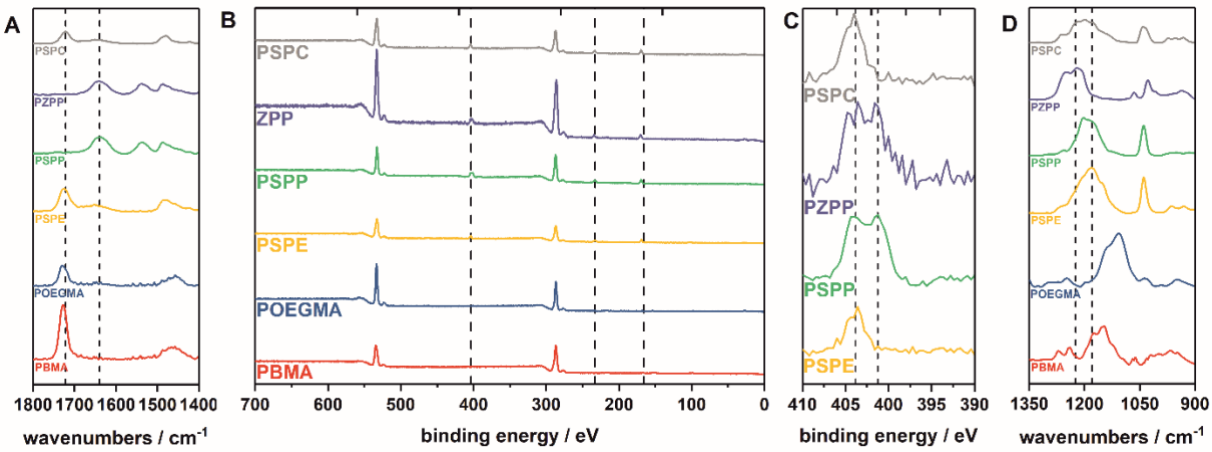

Figure 3. (A) ATR-FTIR spectra of the polymer coatings in the region of ester and amide vibrations. (B) XPS spectra of the polymer coatings showing the N 1s, S 2s, and S 2p binding energy region. (C) Magnified N 1s binding energy region in the XPS spectra of the zwitterionic coatings PSPE, PSPP, PZPP and PSPC. (D) ATR-FTIR spectra of the polymer coatings in the region of the sulfonate and sulfate stretching vibrations.

To verify whether the polymers were attached to the surfaces, they were analyzed by ATR-FTIR spectroscopy and XPS analysis (Figure 3). Figure 3A shows the ester and amide region of the IR spectra of all polymer coatings. As expected, PBMA, POEGMA, PSPE and PSPC reveal signals in the ester region (1720 cm^{-1}), while PSPP and PZPP show vibrational bands in the amide region (1630 cm^{-1}). Figure 3B shows the XPS survey spectra of the polymer coatings. The nitrogen signals occur at 400 eV, and the sulfur signals at 233 eV and 162 eV. While no nitrogen signal is observed for the uncharged controls PBMA and POEGMA, the polyzwitterions PSPE and PSPC display one, and PSPP and PZPP two nitrogen signals (Fig. 3C). The single nitrogen peaks for the polymers PSPE and PSPC originate from the cationic ammonium group, while the additional signal in the case of PSPP and PZPP originates from the methacrylamide repeat units. PSPE and PSPC reveal very similar IR and XPS spectra as they are strcural pseudo-isomers, thus containing the same functional chemical groups. Figure 3D shows the sulfonate and sulfate regions of the ATR-FTIR spectra. The S=O stretching

vibration occurs at 1180 cm^{-1} in the case of the sulfonates PSPE, PSPP and PSPC, while it is shifted to higher wavenumbers of 1218 cm^{-1} for the sulfate group of PZPP. Thus, IR distinguishes the sulfo- and sulfabetaine type polyzwitterions, revealing the presence of the sulfonate or the sulfate group in the different polymers. The combined data of XPS and ATR-FTIR provide clear evidence that the specific polymers were deposited successfully onto the surfaces.

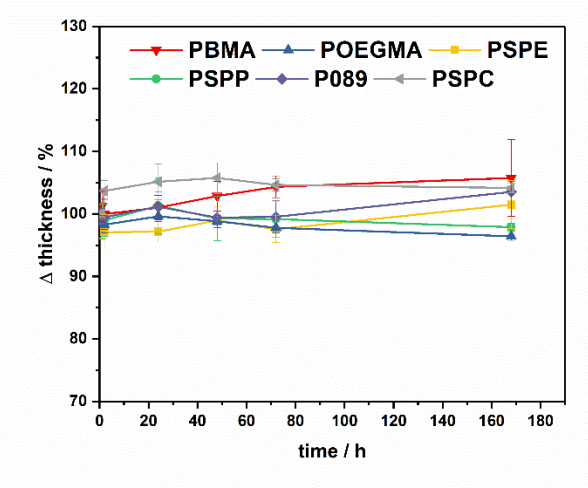

Figure 4. Stability of the coatings in salt water: change of coating thickness after immersion in salt water [SW, pH 8] for 1 h, 2 h, 1 d, 2 d, 3 d, and 7 d at room temperature, as determined by spectroscopic ellipsometry. The thicknesses are compared to the values of the pristine samples. Each point is the average of three independent measurements. Error bars show the standard error of the mean.

Prior to biological testing, the stability of the polymer coatings was tested by immersion in salt water (SW), a model medium for sea water which contains its essential salts but is devoid of any organic components that might cause conditioning layers.[54] The surfaces were immersed for up to 7 days, and the thickness changes were determined by spectroscopic ellipsometry (Figure 4). All polymers retained their thickness over time (≥96%) and were stable even after 7 days' immersion in SW. This finding is in good agreement with a recent long-term study on

the hydrolytic stability of the polymers in solution.[48] As all biological measurements lasted less than 36 h, the polymer coatings could be used for biological testing without risk of degradation.

Protein adsorption. The resistance of the polyzwitterion coatings to protein adsorption was evaluated by surface plasmon resonance spectroscopy (SPR), using identically prepared coatings of PBMA and POEGMA as, respectively, negative and positive controls (Figure 5). We selected a set of proteins with varying net charge and size for the tests. The amphiphilic BSA (pI 4.8[58]) and fibrinogen (pI 5.9[58]) are negatively charged at the pH of the buffer solution of 7.4, while lysozyme (pI 10.9[59]) is positively charged. After baseline stabilization, proteins were injected for a dynamic 10 min incubation phase, which was followed by a 15 min dynamic rinsing step. The obtained sensorgrams are exemplarily shown for the BSA experiments in Figure 5A. SPR responses µRIU were calculated from the angular positions of the plasmon resonance signal before injection of the protein and after the rinsing step (Figure 5B).

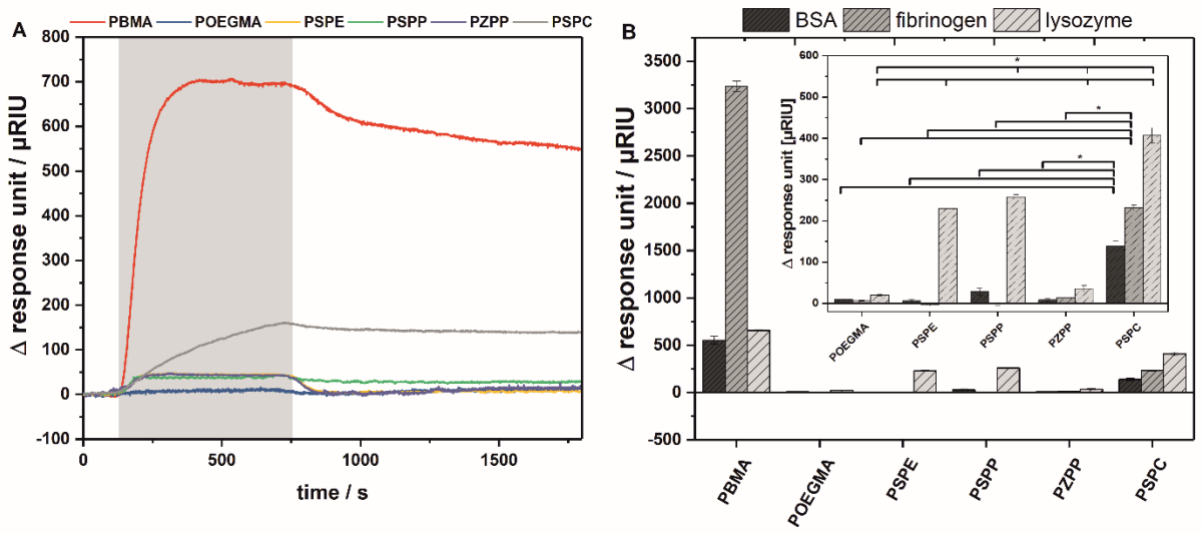

Figure 5. (A) SPR sensorgrams of the polymer coatings exposed to the protein BSA. The white regions indicate injection of buffer solution, the grey region injection of 1 mg/mL BSA solution. (B) Fouling behavior (irreversibly bound protein, average of at least three replicate measurements per protein) of the polymer coatings against model proteins BSA, fibrinogen, and lysozyme. The inset magnifies the data omitting the negative control PBMA. Error bars

represent the standard deviation. A significant discrimination between PBMA and POEGMA and all polyzwitterions is observed at a significance level of 0.05 by using an ANOVA with post hoc Tukey test.

Large amounts of protein were throughout adsorbed onto the hydrophobic polymer PBMA (negative control), but only marginal amounts onto the POEGMA coating. All four zwitterionic coatings reduced the adsorption of the three proteins strongly, too, providing good fouling protection. Nevertheless, we note significant differences between the performances of the individual polyzwitterions, although their chemical structures are very similar and the densities of zwitterionic groups in the polymers are virtually the same. Moreover, the relative performances of the polyzwitterions varied for the different proteins. Polyzwitterions PSPE, PSPP and PZPP were highly inert against the negatively charged proteins, very similar to POEGMA. In contrast, the positively charged lysozyme adsorbed to a certain extent onto the polysulfobetaines PSPE and PSPP, while the coating of polysulfabetaine PZPP rejected lysozyme nearly as effectively as the POEGMA film. The lower inertness of zwitterions compared to positively charged proteins is in agreement with other reports .[35,60] Accordingly, the inertness of polyzwitterion PZPP against all tested proteins matches the inertness of POEGMA. In contrast, polysulfobetaine PSPC, which is a pseudo-isomer of PSPE differing in the orientation of the zwitterion anchored to the backbone, accumulated considerably larger amounts of proteins than the other polyzwitterions. Still, PSPC reduced protein attachment markedly compared to PBMA, namely by 75% for BSA, by 97% for fibrinogen and by 38% for lysozyme. An ANOVA with post-hoc Tuckey test verified that the protein rejection ability of the zwitterionic coatings of PSPE, PSPP and PZPP that all bear linear zwitterionic side chains for all three proteins was significantly higher (significance level $p < 0.05$) than for PSPC bearing a Y-shaped zwitterions in its side chain. This remarkable finding demonstrates the importance of the correct presentation of the zwitterions within the polymers for their anti-fouling

performance. As polysulfobetaines PSPE and PSPC differ *a priori* only in the orientation of the betaine attachment to the backbone, this suggests that either the lack of a spacer group separating the betaine moiety from the polymer backbone is responsible for the markedly lower performance of PSPC, or that perpendicularly attached zwitterionic moieties independent of the nature of the anionic group (i.e., for both sulfo- and sulfabetaines) were more effective to achieve protein resistance than the newly made Y-shaped attached analogue. The latter proposed explanation would coincide with recent findings that the orientation of ammoniophosphate zwitterions, either in the form of phosphatidylcholines or of choline phosphates, markedly affects the interaction of such zwitterionic materials with cells and proteins.[61–63] Hence, it is not only the presence and density of zwitterionic moieties in protective polymer coatings, which defines their anti-fouling behavior, but also the precise nature of the zwitterionic group (*cf* polysulfobetaine PSPP vs. polysulfabetaine PZPP) as well as the orientation of zwitterion attachment (perpendicular in PSPE vs Y-shaped in PSPC) are highly important. Moreover, even the detailed chemical structure of the anchoring carbonyl moiety (namely ester in PSPE vs secondary amide in PSPP) cannot be neglected when optimizing polyzwitterions, even if the effect of this latter molecular variable is much smaller than of the former two. An increased adsorption level for BSA is found on PSPP coatings as compared to PSPE. One may speculate whether H–bonding interactions of the proteins with the secondary methacrylamide groups in PSPP are responsible for this difference. In any case, the comparison of the set of polyzwitterions reveals, that the precise design of their structure may boost their generally high protein resistance even further.

The performance of all polymers with linear zwitterionic side chains differed significantly for all three proteins from that of hydrophobic PBMA and the Y-shaped zwitterion PSPC ($p<0.05$). The difference between PSPE and PSPC, which are distinguished by the orientation of the betaine attached to the backbone, suggests that polymethacrylates with perpendicularly attached

sulfo- and sulfabetaines are more effective to achieve protein resistance than the new Y-shaped analogue. Alternatively, the lack of a spacer group separating the betaine moiety from the polymer backbone may be responsible for the lower performance. The different extent of adsorption of the net positively charged protein lysozyme, when comparing PSPP and PZPP, suggests a higher tendency to attach to surfaces decorated by sulfobetaines than their sulfabetaine analogues (e.g. PZPP). Interestingly, this finding is opposite to theoretical predictions.[47]

Microfluidic attachment assay of the marine diatom *Navicula perminuta.*

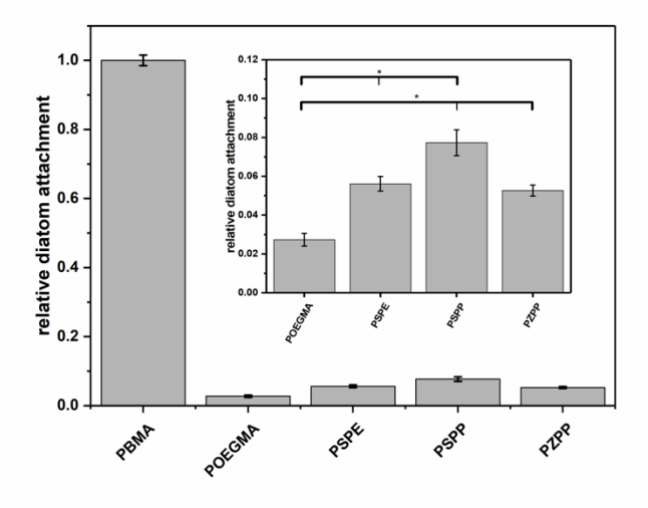

Figure 6. Diatom attachment on polyzwitterion coatings relative to PBMA. The dynamic attachment assay was performed over 90 min at a wall shear stress of 0.14 Pa. Brackets indicate statistically significant differences (*, $p<0.05$) and error bars are the standard error (n=3).

In addition to protein resistance, we investigated the initial attachment of two typical marine fouling organisms, namely of the diatom *Navicula perminuta* of the green alga, *U. linza*, on the polymer coatings. Due to its notably lower protein resistance, polysulfobetaine PSPC was not included in these assays. As the diatom species is non-motile in the water column, the attachment process of *Navicula perminuta* was carried out under dynamic conditions.[56] The relative diatom densities provide a measure of the initial attachment strength of the diatoms and

allow an estimation of how easily diatom biofilms can be removed from the coatings. Figure 6 summarizes the obtained dynamic accumulation data of *N. perminuta* on all investigated polymers. The data were normalized to PBMA following previously established protocols.[56] As in the protein adsorption test, the highest number of settled diatoms was observed on the hydrophobic control PBMA. In comparison, for the hydrophilic control POEGMA and all three polyzwitterions, settlement was significantly reduced ($p < 0.05$). Within the set of polyzwitterions, the trend showed some similarities with the protein assays (Figure 5b). The polymethacrylates PSPE and PZPP reduced settlement to a greater extent ($p < 0.05$) than the polymethacrylamide PSPP. The slightly more effective performance of polysulfabetaine PZPP compared to PSPE may be apparent only, as the difference is not statistically significant.

Attachment of zoospores of the green algae *Ulva linza.*

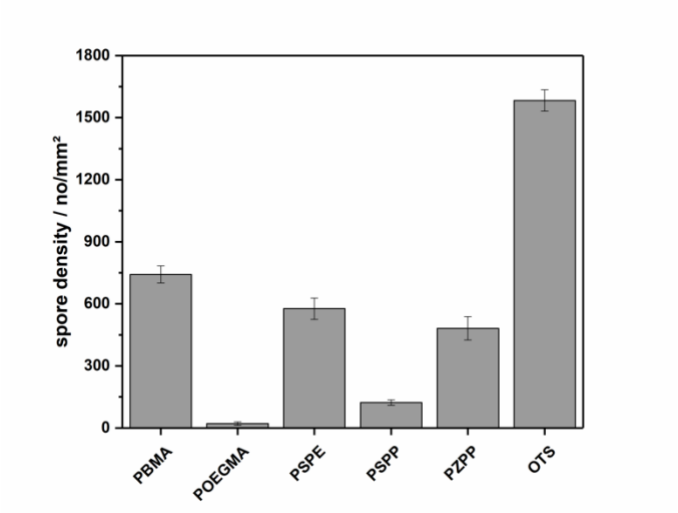

Figure 7. Density of attached zoospores of the green alga Ulva linza after 45 min of incubation. Error bars indicate the 95% confidence limits (n=3).

In addition to the dynamic diatom attachment experiment, the settlement of zoospores of the green alga, *U. linza*, was tested. In contrast to diatoms, the zoospores can actively swim and select surfaces that are suitable for settlement[57,64]. Again, the resulting settlement densities (figure 7) depend on the specific polymer surface. Spore attachment was highest on the

hydrophobic control coatings of n-octadecyltrichlorosilane (OTS) and PBMA, while the zwitterionic coatings markedly reduced the density of settled zoospores. Most effective were the oligo(ethylene glycol)-based coatings, on which the spore density was reduced to 1 % compared to the control OTS, and to 3 % compared to PBMA. Next best reductions were encountered for PSPP, with 8 % spore density compared to OTS and 17 % compared to PBMA. In contrast and quite different from the results for the settlement of the diatoms, PSPE and PZPP performed only moderately better than the PBMA control. The differences between the tested coatings were statistically significant ($p < 0.05$).

In all assays, the adsorption of proteins and marine organisms on the hydrophobic reference coatings of PBMA and of OTS was higher than on the zwitterionic coatings. In the case of the settlement of zoospores of *U. linza*, this is in agreement with previous findings that have shown settlement to be high on some hydrophobic surfaces.[8,65] As anticipated, POEGMA resulted in the lowest accumulation in all assays. The excellent antifouling properties of oligo(ethylene glycol)s have been extensively reported in the literature, however, their long term stability is endangered by oxidative degradation of the polyether function.[7,66] Due to their chemical nature, i.e. the lack of ether groups, the same oxidative degradation is absent in polyzwitterionic methacrylates. The stability in salt water was confirmed by spectroscopic ellipsometry and contact angle goniometry which gave no indication for any instability of the coatings after 7 days. A more extensive stability test was previously carried out by NMR spectroscopy which confirmed the stability of the zwitterionic polymers.[47] The studied polyzwitterions lowered the accumulation of foulants effectively in comparison to the hydrophobic controls in both, the protein and diatom assay, and thus exhibited promising antifouling properties. The observed differences in the properties of the various polyzwitterions, which also differ for the different fouling species, are remarkable. The results demonstrate that high zwitterion density alone is not decisive for the anti-fouling effectivity, but that an appropriate design may boost the general

anti-fouling capability. Clearly, the precise polymer structure and architecture is important for the performance of the polymer coatings. Also, 'the' universal optimum structure has yet to be discovered. The differences found between the sulfobetaine polymethacrylate analogous PSPE and PSPC underline the importance of the orientation of the betaine groups within the polymer architecture. The parallel attachment of the betaine group to the polymer backbone in SPC seems less favorable than its perpendicular anchoring. The reason however, has to be clarified in future studies, as the missing spacer group between polymer backbone and the betaine moieties might be alternatively responsible for the effect. PSPE as polymethacrylate and PSPP as polymethacrylamide only differ in the linker groups attaching the identical betaine motif to the backbone. This apparently small structural difference affected nevertheless the antifouling properties of the polymer. While PSPE was more effective in the protein and diatom assays, PSPP performed more effectively in the *U. linza* assay. Although in the literature, polyzwitterions have been most frequently prepared based on polymethacrylate[4,15,25,36,60,67] as well as on polymethacrylamide backbones[21,36,68,69] yielding generally low fouling surfaces, a direct comparison between both convenient linkers with respect to the antifouling properties has so far been missing. The polyzwitterion PZPP with the sulfate group was particularly effective at reducing the adsorption of all the proteins assayed, irrespective of mass and net charge, and it outperformed all other zwitterionic polymer coatings in this study. The lysozyme and the dynamic diatom assays showed similar trends. This finding is remarkable because it exemplifies the importance of the precise ionic groups present in the zwitterionic moieties on the one hand, and contradicts previous predictions from molecular modeling on the other hand.[47] However, the effectivities of PSPE and PZPP coatings against diatom attachment did not differ significantly.

The comparison between PSPP and PZPP is instructive as both polymers share the polymethacrylamide backbone and only differ in the nature of the anionic group of the betaine

motif, namely they carry a sulfonate or a sulfate group. Both polymers showed a high inertness against the positively charged proteins BSA and fibrinogen which is comparable to the inertness of POEGMA. In contrast, the positively charged protein lysozyme attached considerably more strongly to the polysulfobetaine PSPP than to PZPP. Thus, the sulfabetaine PZPP presented in general very good antifouling potential. The dynamic diatom accumulation experiments followed the trends in the protein adsorption experiments and attachment on PSPP exceeded the one on PZPP. The zoospore accumulation assay showed a different trend, in which settlement was lower on PSPP compared to PZPP.

In general, the data are in line with recent experiments on self-assembled monolayers in which the influence of sulfonate, carboxylate[8], and phosphonate[19] on the attachment of proteins and marine organisms was explored. Our findings that several sulfobetaine polymers effectively reduce fouling agrees very well with the fouling reduction found on the sulfonate-containing self-assembling monolayers.[8] The results also support the previous finding of the Jiang group about the remarkable resistance of polysulfobetaines against attachment of proteins[4] or barnacle cyprids[18] and the experiments of Yang et al showing a high effectiveness of polyvinyl alcohols functionalized with sulfobetaines against green algae and marine diatoms.[25,38] As reported by Finlay et al., SBMA polymers show very good fouling release performance against *Navicula incerta*.[40] This weak attachment of marine fouling species is maintained when the zwitterionic SBMA components are embedded into siloxane-polyurethane matrix.[41]

Our observation that the connection to the backbone, i.e. length of the aliphatic spacer and/or connection via amide or ester, affects adhesion of fouling organisms is in good agreement with the recent comparison of biofouling on zwitterionic polymers that were constructed using backbone chemistries, namely, polyacrylate and surface grafted polymethacrylate.[42] In our study, we find that the polysulfabetaine is slightly more effective than the analogous polysulfobetaine.

Summary and Conclusion. We developed a set of custom–made sulfo- and sulfabetaine bearing zwitterionic copolymers containing a photo–crosslinker to obtain stable and homogenous low-fouling surfaces. The spin–coated polymer films were fully characterized by water contact angle goniometry, AFM, ATR-FTIR and XPS. High quality coatings can be prepared with a high stability in different media. Importantly, we show that the antifouling properties of a polyzwitterion depend sensitively on the chemical nature of the functional groups. The comparison of a set of polyzwitterions based on sulfonate and sulfate groups revealed that the polymer connection and/or spacer between the zwitterion and the polymer is relevant, and that zwitterions containing a sulfate provided mostly better fouling resistance than zwitterions containing a sulfonate. The sulfate zwitterion PZPP reduced the attachment of lysozyme and *N. perminuta* considerably with respect to its sulfonate zwitterion analog, PSPP, but was less effective in the particular case against the attachment of zoospores of *U. linza*. We also showed that the arrangement of the charged groups in the side chains has a high impact on the protein adsorption. The non-linear orientation of the zwitterion led to increased protein adsorption compared to a linear zwitterion one.

Our results confirm the general notion that polyzwitterions reduce the attachment of foulers on surfaces and that betaines are promising hydrophilic components for future antifouling coatings. The systematic variations of the polyzwitterion structure together with the comparative tests investigating diverse foulers, proteins as well as organisms, resulted nevertheless in a rather diversified picture for anti-fouling coatings. On the one hand, there seems to be no universal optimum structure providing best anti-fouling performance in all situations. On the other hand, the anti-fouling effectivity is modulated by the precise chemical structure of the polymer system employed. Even if no reliable guiding rules can be offered currently, our findings demonstrate that not only the chemical nature of the zwitterionic moiety incorporated, but also the attachment geometry of the zwitterionic side chains and even the

groups linking the side chains to the polymer backbone are valid parameters to influence the anti-fouling behavior. Presumably, there will be other useful molecular variables, too. Also, the degree of cross-linking will most probably affect the antifouling performance. Realizing that up to now most zwitterionic polymers employed for anti-fouling have been based on a surprisingly small pool of monomers, i.e., SPE, SPP, phosphorylcholine methacrylate ("MPC") and two carboxybetaine methacrylates ("CBMA"),[20] new polyzwitterion structures are therefore needed to boost their anti-fouling behavior for general purposes as well as for specific problems.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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